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Resistance of *Leishmania infantum* to allopurinol is associated with chromosome and gene copy number variations including decrease in the S-adenosylmethionine synthetase (*METK*) gene copy number

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ABSTRACT

Leishmania infantum is one of the causative agents of visceral leishmaniasis (VL), a widespread, life-threatening disease. This parasite is responsible for the majority of human VL cases in Brazil, the Middle East, China, Central Asia and the Mediterranean basin. Its main reservoir are domestic dogs which, similar to human patients, may develop severe visceral disease and die if not treated. The drug allopurinol is used for the long-term maintenance of dogs with canine leishmaniasis. Following our report of allopurinol resistance in treated relapsed dogs, we investigated the mechanisms and markers of resistance to this drug. Whole genome sequencing (WGS) of clinical resistant and susceptible strains, and laboratory induced resistant parasites, was carried out in order to detect genetic changes associated with resistance. Significant gene copy number variation (CNV) was found between resistant and susceptible isolates at several loci, including a locus on chromosome 30 containing the genes *LinJ.30.3550* through *LinJ.30.3580*. A reduction in copy number for *LinJ.30.3560*, encoding the S-adenosylmethionine synthetase (*METK)* gene, was found in two resistant clinical isolates and four induced resistant clonal strains. Using quantitative real time PCR, this reduction in *METK* copy number was also found in three additional resistant clinical isolates. Furthermore, inhibition of S-adenosylmethionine synthetase encoded by the *METK* gene in allopurinol susceptible strains resulted in increased allopurinol resistance, confirming its role in resistance to allopurinol. In conclusion, this study identified genetic changes associated with *L. infantum* resistance to allopurinol and the reduction in *METK* copy number identified may serve as a marker for resistance in dogs, and reduced protein activity correlated with increased allopurinol resistance.

1. Introduction

Visceral leishmaniasis (VL) is a debilitating, life threatening disease affecting hundreds of thousands of people annually (Alvar et al., 2012). According to recent WHO estimations, 556 million people are exposed to VL in 12 high-burden countries, with 300,000 cases and 20,000 deaths worldwide annually. The causative agents of this disease are *L. infantum* in South America, the Mediterranean basin, and parts of Europe, North Africa, and Asia; and *L. donovani* in East Africa and the Indian subcontinent (World Health Organization, 2010; Alvar et al., 2012; Gardoni, 2013). A limited number of drugs are available to treat VL, mainly pentavalent antimonials, miltefosine, and amphotericin B in human patients (Freitas-Junior et al., 2012; Monge-Maillo and López-Vélez, 2013). Antimonials, miltefosine and allopurinol are the drugs of choice for dogs, which are the main reservoir for *L. infantum* (Solano-Gallego et al., 2011). Resistance to antileishmanial drugs is well documented in humans (Croft et al., 2006; Berg et al., 2013; Leprohon et al., 2014), but poorly studied in dogs. We have recently described resistance to allopurinol in *L. infantum* isolated from treated dogs with disease relapse (Yasur-Landau et al., 2016). Such resistant parasite strains may promote the spread of infection between dogs, and from dogs to humans (Quinnell and Courtenay, 2009; Yasur-Landau et al., 2016).

Allopurinol is a purine analog with anti-leishmanial activity (Pfaller and Marr, 1974). It is rarely prescribed for human VL (Mishra et al., 2007; Freitas-Junior et al., 2012; Monge-Maillo and López-Vélez,

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2013), but extensively used for long term control of canine *L. infantum* infection, either by itself or in combination with meglumine antimoniate or miltefosine (Solano-Gallego et al., 2011). Based on the induction of allopurinol resistance *in-vitro* in susceptible *L. infantum* isolates from dogs in a previous study, we concluded that a genetic basis for resistance against this drug exists (Yasur-Landau et al., 2017). In the current study, whole genome sequencing was used to detect differences in the genomes of allopurinol-susceptible and resistant clones, and isolates from dogs. The resistant isolates were obtained from naturally-infected clinically relapsed dogs receiving allopurinol maintenance therapy, as well as strains induced *in-vitro* under drug pressure. Consistent phenotype-related changes between allopurinol susceptible and resistant strains were found in ploidy and gene copy numbers. Based on these results, we chose to focus on the role of the enzyme S-adenosylmethionine synthetase in allopurinol resistance.

2. Materials and methods

2.1. Allopurinol-susceptible and resistant strains

Twenty L. *infantum* strains were isolated from dogs showing clinical signs of leishmaniasis (Solano-Gallego et al., 2011; Yasur-Landau et al., 2016), 15 of which were isolated prior to any drug treatment (non-treated group – NT) and 5 after disease relapse during allopurinol treatment (treated relapsed group – TR). Each isolate was identified as *L. infantum* using a real time PCR assay amplifying a part of the parasite's internal transcribed spacer (ITS-1), followed by DNA sequencing (el Tai et al., 2000). Strains from the TR group were allopurinol resistant, showing an average IC_{50} value 4 folds higher compared to that of the NT group (Yasur-Landau et al., 2016). Strains NT1-10 as well as TR1-4 were previously described in detail (Yasur-Landau et al., 2016). Two of the susceptible strains (NT4 and NT5) from non-treated dogs were used in the induction of allopurinol resistance (termed NT4.L and NT5.L), achieved by culturing parasites in increasing concentrations of allopurinol over a course of 5 months and producing allopurinol resistant strains, as previously described (Yasur-Landau et al., 2017). Ten susceptible and 10 resistant strains representing 5 different sampling points in each of the two resistance induction experiments were used in the following experiments, as well as 6 clonal strains derived from the induced cultures of NT4.L and NT5.L on days 28, 104 and 28, 86, respectively (Supplementary figure 1). The latter 6 clonal strains, two resistant and one susceptible each from NT4.L and NT5.L were also submitted for whole genome sequencing (WGS), alongside strains NT10 and NT4, TR2 and TR4, each pair representing the susceptible (low IC_{50}) and resistant (high IC_{50}) phenotype, respectively. Information on all strains used in this study is included in supplementary Table 1. Clones were produced using the hanging drop method, as previously described (Evans and Smith, 1986; Yasur-Landau et al., 2016). Allopurinol susceptibility was determined using a viability assay and expressed as IC_{50} using Prism 5 software (GraphPad Software, San Diego, CA), as previously described (Yasur-Landau et al., 2016).

2.2. DNA extraction and WGS

DNA for WGS was extracted from mid-log phase promastigotes of each of the 10 strains and prepared for whole genome sequencing as previously described (Yasur-Landau et al., 2017). Sequencing was done using 100 bases paired ends reads, on an Ilumina HiSeq2000 platform. Clonal strains NT4.L.r1, NT4.L.r2, NT4.L.s, NT5.L.r1, NT5.L.r2 and NT5.L.s were sequenced at the DNA LandMarks Inc. Laboratory (St.-Jean-sur-Richelieu, Canada), and submitted to GenBank™ under the accession numbers SAMN09079779-84. Isolates MCAN/IL/2012/NT16, MCAN/IL/2009/TR2, MCAN/IL/2011/NT10 and MCAN/IL/2011/TR4 were sequenced at the Wellcome Trust Sanger Institute, UK, and submitted by the Sanger Institute to the European Nucleotide Archive with accession numbers SAMEA1708595-8, respectively.

Raw reads cleaning, trimming and mapping were also done as previously described, using the *L. infantum* JPCM5 genome, chromosomes 1–36 as reference (European nucleotide archive, BioProject PRJNA12658, FR796433 - FR796468). The soap.coverage (version 2.7.7) of the SOAP Short Oligonucleotide Analysis Package (https://www.ncbi.nlm.nih.gov/pubmed/18227114) was used for depth estimation of each chromosome in each sample.

2.3. Data analysis

Analysis of ploidy (number of copies of whole chromosomes) was done for each sequenced strain essentially as described by Rogers et al. (2011). Briefly, for each strain, the averaged median read depth of chromosomes 30, 34, 35 and 36 was set to 2 assuming a diploid arrangement, and median readings for each of the other chromosomes were normalized accordingly. Information including coding sequences, gene annotations and protein products of *L. infantum* was obtained from the TriTrypDB website (Aslett et al., 2010). To determine genomic loci amplification (copy number variation, CNV), the Bioconductor cn.MOPS software version 1.6.7 (Klambauer et al., 2012) was used in the R environment with default settings, WL = 2500 and minWidth = 4.

2.4. Quantitative PCR assays of genes LinJ.30.3560 and LinJ.36.0790

Following the analysis of CNV's in the WGS data, two loci showing a CNV with possible connection to allopurinol resistance were further studied. CNV in loci containing genes *LinJ.30.3550-3580* and *LinJ.36.0790* were further determined using quantitative real time PCR (qPCR) with primers designed to amplify parts of the *LinJ.30.3560* and *LinJ.36.0790* genes. Primers were designed using the NCBI/Primer-BLAST website (http://www.ncbi-.nlm.nih.gov/tools/primer-blast/). DNA of clones NT5.L.r1 and NT4.L.s was used as reference (RQ = 1) for *LinJ.30.3560* and *LinJ.36.0790*, respectively. Forward and reverse primers for the GAPDH control gene (do Monte-Neto et al., 2011) were used to amplify a 226 base fragment of *LinJ.30.2990* and *LinJ.30.3000*. According to the *L. infantum* JPCM5 reference genome there are 2 copies of GAPDH, and no evidence for a change in its copy number was found in the WGS data. Therefore, GAPDH was used as a reference gene for quantitation. Primer sequences are presented in supplementary Table 2. PCR reactions were done in a total volume of 20 μL each, including; 10 μL Fast SYBR Green Master Mix (x2) (Applied Biosystems, Foster City, CA), 2 ng DNA, ultra-pure water and a final concentration of 250 nM of the forward and reverse primers. The StepOnePlus real-time PCR thermal cycler (Applied Biosystems, Foster City, CA) was used with the following thermal profile; initial denaturation for 20 s at 95 °C, followed by denaturation for 3 s at 95 °C and annealing for 30 s at 59 °C for 40 cycles. Amplicons were subsequently subjected to a melt step with the temperature raised to 95 °C for 15 s and then lowered to 60 °C for 1 min. The temperature was then raised to 95 °C at a rate of 0.3 °C per second. Amplification, melt profiles and amplicon relative quantity (RQ) based on analysis of the ΔΔCT values, were analyzed using the StepOne V2.2.2 software (Applied Biosystems, Foster City, CA). Each reaction was repeated twice and an average RQ was calculated.

2.5. S-adenosylmethionine synthetase inhibition experiments

S-adenosylmethionine synthetase is an enzyme encoded by the two copies (*LinJ.30.3560/80*) of the *METK* gene, present in one of the two loci showing possible connection to allopurinol resistance. Its effect on allopurinol resistance was studied using an enzyme inhibition assay. Cycloleucine (1-Aminocyclopentanecarboxylic acid) is an inhibitor of the enzyme S-adenosylmethionine synthetase in bacteria, yeast, rat and human cells in culture (Lombardini and Talalay, 1970; Zhuge and Cederbaum, 2007; Jani et al., 2009). The effect of S-adenosylmethionine synthetase inhibition on the parasite susceptibility to allopurinol